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Minireview

Proteomics strategies for protein identification

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Abstract The information from genome sequencing provides new approaches for systems-wide understanding of protein networks and cellular function. DNA microarray technologies have advanced to the point where nearly complete monitoring of gene expression is feasible in several organisms. An equally important goal is to comprehensive survey cellular proteomes and profile protein changes under different cellular states. This presents a complex analytical problem, due to the chemical variability between proteins and peptides. Here, we discuss strategies to improve accuracy and sensitivity of peptide identification, distinguish represented protein isoforms, and quantify relative changes in protein abundance.

Keywords: Proteomics; Mass spectrometry; Bioinformatics

1. "Top-down" vs. "bottom-up" proteomics

The goal of profiling proteins and peptides represents a complex analytical problem, due to high chemical variability and solubility differences between analytes. Two strategies are top-down proteomics, which analyzes intact proteins, and bottom-up proteomics, which analyzes peptides in proteolytic digests. An established top-down strategy for protein profiling is two-dimensional gel electrophoresis (2DE), which displays changes in protein expression and covalent modification based on protein staining intensity and electrophoretic mobility. To identify proteins of interest, gel pieces are excised, peptides are extracted by in-gel digestion, then analyzed by mass spectrometry [1]. Computer algorithms identify proteins based on peptide mass and fragmentation (MS/MS) information to search protein databases. Several outstanding papers have been published using this methodology to identify novel functions for defined molecules [2–5]. However, 2DE can be limited by detection sensitivity and protein solubility; high abundance proteins generally less than 120 kDa are favored, and integral membrane or basic proteins may be difficult to resolve. Currently, no laboratory has surveyed more than several hundred proteins by 2DE in mammalian cells [6,7], which represents less than 10% of the probable size of a proteome, i.e., the number of expressed ORFs in a given cell type.

More recently, top-down profiling has been carried out using mass spectrometry by full mass analysis of proteins after fractionation using partially selective adsorption matrices (e.g., SELDI), or by multidimensional chromatography, capillary electrophoresis, or free flow electrophoresis to resolve proteins before mass analysis [8–10]. Because proteins are difficult to ionize, detection is limited and identification requires mass spectrometers capable of high resolution (e.g., ~1 ppm) with the ability to fragment large analytes. Successful results have been obtained by top-down FT-ICR MS [11–13] although its routine use for surveying complex samples requires further instrument development.

A protein profiling strategy, variously referred to as "bottom up" shotgun proteomics, multidimensional LC/MS/MS, or multidimensional protein identification technology (MudPIT), involves solution proteolysis of a complex mixture of proteins, followed by chromatographic separation of peptides prior to MS/MS sequencing (Fig. 1) [13,14]. Often, protein separation and enrichment is carried out before digestion, for example by protein chromatography or organelle purification. A variation of this approach separates proteins by SDS–PAGE, followed by in-gel digestion of proteins which comigrate in gel slices within narrow mass ranges, and subsequent multidimensional LC/MS/MS [15]. Improved software in current mass spectrometers allows peptide sequencing by data-dependent data acquisition, in which ions are automatically selected and fragmented by MS/MS, enabling thousands of spectra to be collected in a single reversed phase analysis [16–18].

The viability of shotgun proteomics for global protein profiling was first shown in a study identifying more than 1400 proteins from *Saccharomyces cerevisiae*, including low abundance proteins [18]. Recent studies have identified 1504 (25% of ORFs) from *S. cerevisiae*, 1910 proteins in *Deinococcus radiodurans* (61% of ORFs), 2415 proteins in *Plasmodium falciparum* (46% of ORFs), and 5130 proteins (15% of ORFs) in human erythroleukemia cells [19–22]. In addition, large projects have been initiated to comprehensively catalog the protein content of human tissues and fluids [23,24].

Protein profiling in higher eukaryotes presents a more complex analytical problem than yeast or bacteria, due to greater genome sizes and numbers of proteins specified, longer protein sequences, wider protein concentration ranges, and greater variability in alternative splice and covalently modified forms. Mammalian protein profiling must address several problems in order to improve (i) accuracy and sensitivity of peptide identification, (ii) discrimination between protein isoforms, (iii) protein quantitation, and (iv) analysis of covalent modifications. All are consequences of the larger genome sizes of mammalian

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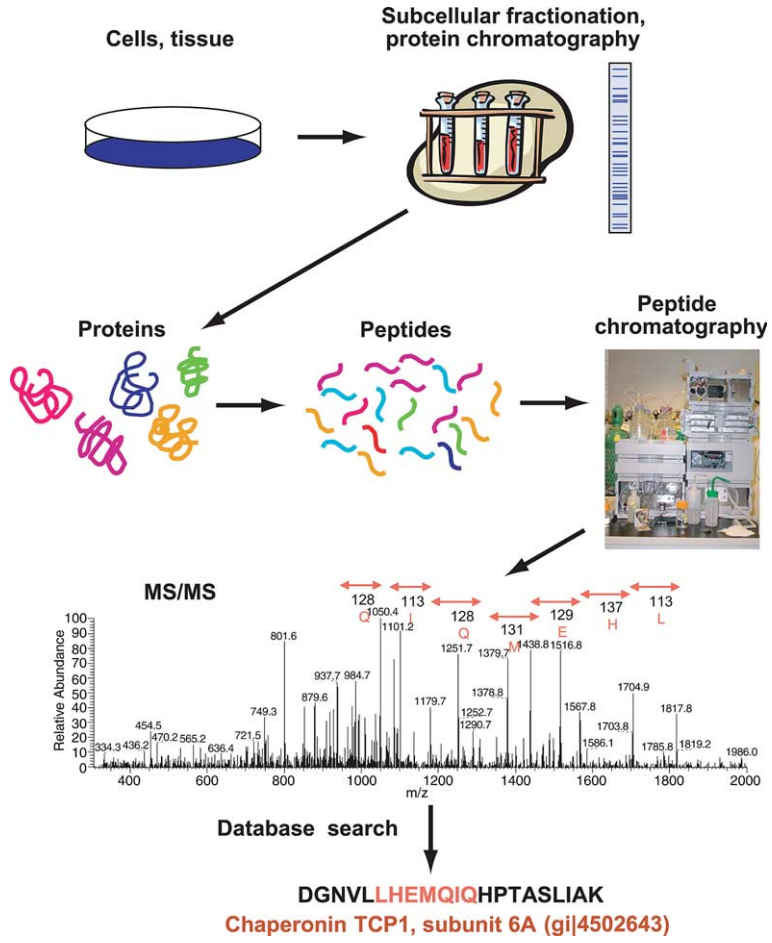

Fig. 1. Protein profiling by shotgun proteomics. Complex mixtures of proteins are proteolyzed in solution, and resulting peptides are simplified by chromatographic separation prior to MS/MS sequencing. Often, protein separation and enrichment is carried out before digestion, for example by protein chromatography, SDS–PAGE, or organelle purification.

cells, which impact spectral collection and data reduction. Here, we discuss approaches to improve accuracy and sensitivity of peptide identification, distinguish represented protein isoforms, and quantify relative changes in protein abundance.

2. Accuracy and sensitivity in search programs

To identify peptide sequences from MS/MS spectra, the most successful search programs (e.g., Mascot, Sequest [25–28]) compare observed fragment ions against theoretical ion mass, and/or determine the statistical likelihood of generating observed fragment ions from candidate peptide sequences represented in a protein database. A scoring method is then used to rank the various peptide candidates, based on similarity between theoretical and observed spectra. A common approach for validating peptide matches is to accept all assignments with scores above a certain threshold. Thresholds for high confidence acceptances may be determined by searching the same datasets against a ''randomized'' protein database, created by inverting each protein sequence contained in the normal database [29,30]. Each assignment is therefore incorrect, providing an estimate of false positives; from this data, threshold values can be set which maintain false positives below a desired level.

Fig. 2 shows a histogram of scores from human peptide sequences. Scores obtained by searching a normal human protein database show a biphasic distribution, with the majority of spectra with scores between 1 and 2.5, and a shoulder at high scores representing high confidence assignments. The score distribution of incorrect assignments obtained by searching a randomized database can be seen to coincide with the major peak of normal assignments. From this analysis, scores above XCorr = 2.55, 3.39, or 3.78, respectively, for MH^{1+}, MH_2^{2+}, or MH_3^{3+} ions produce false positives <0.5%. However, when the distribution of correctly assigned spectra is estimated using data collected with protein standards, more than half of the correct assignments receive low scores and are rejected [22].

High false negatives limit the sensitivity of peptide detection, which is related to how many spectra can be assigned accurately to peptide sequences. Typically, useful peptide information is obtained from only 8% to 25% of the data collected. For example, extensive analyses to classify MS/MS spectra in a human dataset showed that Sequest or Mascot searches identified 24% of the spectra. Of the remaining 76%, two-thirds were products of non-specific proteolysis, fragment ions, weak or incorrectly formed data files, and modified peptide artifacts, which provide little protein identification information. The remaining MS/MS spectra were identifiable and of good quality, but could not be unequivocally validated by the scoring

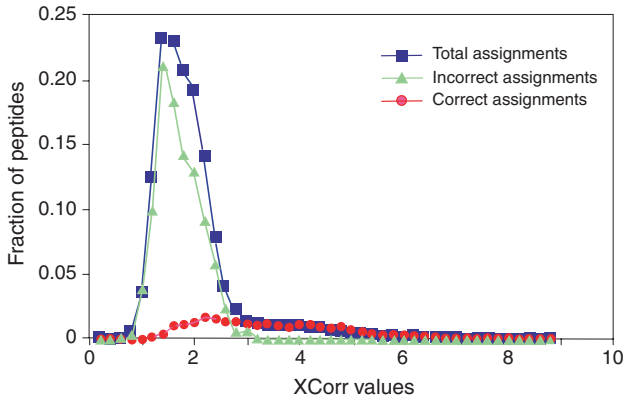

Fig. 2. XCorr distribution for MH_2^{2+} peptides in a shotgun proteomics dataset of ~10 000 MS/MS spectra human proteins. MS/MS data files were searched against a normal human protein database using the Sequest program (squares). Searching MS/MS spectra against a database of randomized sequences provides a score distribution of false positive assignments (triangles). From this distribution, threshold values can be set which maintain false positives below a desired level, so that peptide sequence assignments are almost always correct. However, the distribution of MS/MS spectra with correct sequence assignments confirmed by manual analysis (circles) show that approximately equal numbers of correctly assigned spectra typically score above and below threshold. Spectra for correct sequences scoring below threshold may be misassigned when incorrect sequences in the database yield higher scores.

methods of Sequest or Mascot. This illustrates how setting high thresholds to minimize false positives leads to increased numbers of false negatives. Additional strategies are needed to improve accuracy without sacrificing sensitivity.

3. Improving search strategies

Various approaches generate probability rankings based on several parameters generated by search programs [29–33]. A program implementing a linear discriminant strategy, named Peptide Prophet, developed coefficients to weight different Sequest scores, using Bayesian statistics to develop a probability ranking for sequence identifications [31]. Strengths of the program are that multiple indices were combined to improve the discrimination of assignments. A potential weakness is that the two strongest probability determinants (XCorr and ΔCN) are positively correlated in datasets of mammalian proteins, which may introduce biases in probability values, thus often a large fraction of correct assignments are not validated. Another method to improve confidence of sequence assignments uses exact peptide mass measurements to validate peptide assignments [13,20]. Determining tryptic peptide masses to an accuracy of ~1 ppm restricts the number of possible matches to database sequences, and when combined with filters based on reversed-phase chromatography elution [34], peptides in prokaryotic proteins could be determined with minimum ambiguity. Exact mass measurements may be less powerful for mammalian systems due to greater database size and numbers of covalent modifications, and the FT-ICR MS instrumentation required is expensive and not widely available. Using this method, 61% of ORFs in *D. radiodurans* were identified [20], with >90% of ORFs in updated datasets.

Significant improvements in peptide validation can be gained by incorporating peptide chemical information into data reduction, which current programs do not consider extensively. Filters based on peptide chromatography on ion exchange or reversed phase HPLC have proven successful. Hydrophobicity indices have been used to predict peptide elution on RP, by calculating retention times of individual amino acids, either by linear regression or using a neural net machine learning approach [34,35]. Combining these with exact mass measurements improved the accuracy of bacterial protein identification, illustrating how independent inputs with low discrimination individually produce high discrimination in combination. This principle is used by an algorithm, MSPlus [22], which evaluates consensus between search programs (Sequest and Mascot), reducing false negatives by allowing scoring thresholds to be bypassed. Filters then evaluate the numbers of basic residues on peptides and test their consistency with SCX elution behavior, together with a RankSp parameter from Sequest. MSPlus increases data capture compared to threshold strategies, while maintaining low false positive and false negative assignments and high reproducibility in protein identification.

Although rules based on chemistry improve identification of correct sequences, sensitivity is still limited when search programs produce incorrect assignments that are later rejected. In our analyses of human datasets, ~20% of spectra are of good quality with identifiable peptide sequences, but receive higher scores for incorrect sequences. Large databases increase the frequency of misassignments. In an experiment searching 2117 spectra against either the human IPI protein database (48 000 entries) or the same database without specifying trypsin cleavages in the search (increasing the effective database size by >10-fold), 233 correct sequence assignments were replaced by incorrect assignments using the larger database [22]. Thus, sequence inaccuracy is a more serious problem for mammalian systems than yeast or bacteria, highlighting an important caveat when applying methods developed with yeast to mammalian datasets. Information capture can be optimized by minimizing the effective database size, specifying trypsin, and ignoring covalent modifications. From a restricted set of represented proteins, datasets can then be re-searched for modified peptides and non-tryptic cleavages.

Perhaps the ideal solution for better discrimination and accuracy in sequence identification is to improve the prediction of gas phase peptide fragmentation behavior. Statistical analyses of MS/MS datasets have led to empirical rules for cleavages at different peptide bonds in ions with different charge states. These support a general mechanism for peptide bond cleavage which involves transfer of a proton to a peptide bond by vibrational motion, followed by formation of an oxazolone b ion and y ion, via attack of the backbone carbonyl oxygen at the $n-1$ position [36–40]. Some bonds are particularly labile and specific chemical mechanisms have been proposed which account for these enhanced cleavages, such as those C-terminal to Asp/Glu and His and N-terminal to Pro [38–40]. A "mobile proton" model has been proposed to explain fragmentation behavior, in which peptides are classified as "mobile" when the number of protons exceed the total number of basic residues (Arg, Lys, His), "partially mobile" when the number of protons exceed the number of Arg residues but are equal to or less than the total number of basic residues, and "non-mobile" when the number of protons is equal to or less than the

number of Arg residues (available protons are strongly associated with Arg residues, precluding their migration to other residues). Thus, cleavage C-terminal to Asp/Glu/His/Arg/Lys is favored by the ability of these side chains to donate a proton, yielding MS/MS spectra with few fragment ions and strong bias towards these residues. Enhanced cleavage N-terminal to Pro is explained by the greater basicity of the Pro secondary amine, facilitating proton acceptance during cleavage.

Recently, a kinetic model based on classical kinetics and the mobile proton hypothesis was developed to quantitatively simulate peptide MS/MS fragmentation generated in a 3D ion trap mass spectrometer [41]. This developed a mathematical expression describing rate constants of peptide bond fragmentation based on intrinsic rates determined by adjacent amino acids and proton affinities, and also included other reactions, e.g., dehydration, deammoniation, C-terminal rearrangements, and loss of CO. A training set of 3D ion trap MS/MS spectra was used to fit ~400 parameters defining these rate constants. Simulations using the optimized parameter set agreed well with observed spectra for MH^{1+} and MH_2^{2+} ions up to 2000 Da. A similarity score for overlap between theoretical and observed spectra provided significantly better discrimination than XCorr or Mowse. Thus, modeling gas phase fragmentation based on simple chemical principles greatly improved accuracy and should improve sensitivity of protein detection by increasing usable data that can be extracted.

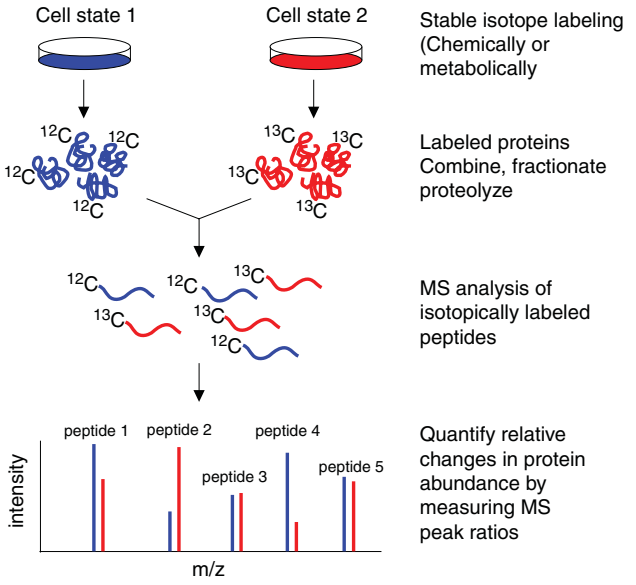

Fig. 3. Relative changes in proteins quantified by stable isotope labeling. Proteins from different samples are differentially tagged with isotopically labeled moieties, which include amino acids incorporated by metabolic labeling, or chemical adducts coupled covalently following cell disruption. Differentially labeled proteins are mixed and proteolyzed, and peptides are analyzed by mass spectrometry. Relative changes in protein abundance are reflected by ratios of peak intensity for matched isotopically labeled peptides.

4. Protein isoform redundancy

An important consideration when identifying proteins from shotgun proteomics datasets is distinguishing protein isoforms. Ambiguity ensues when a given peptide sequence is found in more than one protein entry, a common occurrence. Different protein entries may be assigned variably to spectra representing the same peptide sequence, which has the effect of inflating the protein counts and/or failing to represent protein forms that are present in favor of another containing the same sequence. Programs such as DTAselect and Protein Prophet [32,42] resolve many of these ambiguities, although mass neutral mutations may be overlooked. The program, Isoform Resolver, uses an alternative peptide centric database strategy in which the primary key is each unique peptide sequence, and all proteins associated with each sequence are secondary entries [22]. Improved accuracy of protein identifications from peptide sequences is obtained by carrying forward only the peptide sequence from the search results, and considering all possible mass neutral replacements as possible alternatives for the assignment. Proteins are reconstructed by choosing peptide alternatives in a way that minimizes protein counts. In one analysis, minimizing protein counts that account for the observed peptides led to a ~24% reduction from the number of proteins identified by Sequest or Mascot.

5. Protein quantitation

Quantifying changes in protein abundance between samples is a key goal of shotgun proteomics. Current methods are based on isotope or mass tag labeling of peptides (Fig. 3), in which different samples to be compared are covalently labeled with stable isotope-labeled moieties (e.g., ^{12}C vs. ^{13}C-ICAT reagents, methylisothiourea, ^{16}O vs. ^{18}O-labeled water), or metabolically labeled with isotopically distinguishable amino acids (e.g., ^{12}C vs. ^{13}C-Arg or Lys) [17,43–46]. Proteins from each sample are mixed and relative quantification is determined from the ratio of intensities between the differential isotopically labeled peaks. Shotgun proteomics coupled with peptide quantitation by isotope labeling has been used successfully in many applications to reveal proteomic changes in response to signaling and cell regulation. For example, of 528 proteins surveyed in rat fibroblasts, one-third showed >2-fold changes in protein abundance in response to c-myc signaling, including many with regulatory functions in cell metabolism, adhesion, and morphology [47]. However, such methods are still somewhat limited by relatively low sensitivity in mammalian systems [46]. For example, most of the proteins detected by chemical or metabolic isotope labeling methods in recent publications are relatively abundant, numbering in the hundreds of proteins. Obstacles in chemical labeling methods that require further refinement are unexpected chemistries as well as K_m effects where low abundance peptides do not react to completion or are poorly recovered after affinity enrichment. Metabolic labeling reduces these problems, but cannot be applied to non-cultured cells, such as clinical tissue or serum samples. Both approaches have problems in resolving isotope peaks for larger peptides and require identification of both labeled forms of a peptide.

Promising alternative methods for quantitation involve label-free approaches. Measurement of peptide ion intensities from LC/MS profiles is often used in drug metabolism studies and to estimate stoichiometry of covalently modified peptides. Recently, the feasibility of quantitation from direct measure-

ments of MS peak intensities was reported in quantifying standard curves of proteins added to serum samples [48,49]. Statistical modeling of peak intensities demonstrated good correlations between relative peak intensities of peptide ions and relative protein abundances. A second method counts the total number of MS/MS spectra of peptides in a protein (spectral counts), which was shown to be linearly related to protein abundance in tests of standard proteins added to yeast extracts [50]. With controls for normalization between runs, label-free quantitation may offer a simpler approach for analysis. Spectral sampling should also enable ranking of different proteins by their relative abundances, providing information that other methods cannot achieve.

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